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(54) Title: METHOD FOR DIAGNOSIS AND TREATMENT OF CANCER

(57) Abstract

A method for diagnosing and treating tumors especially brain, liver, lung, esophageal, stomach, colon, rectal, thyroid, and lymphona tumors using antagonists, antibodies and antisense nucleotides to secretory protein–9.

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METHOD FOR DIAGNOSIS AND TREATMENT OF CANCER

BACKGROUND OF THE INVENTION

Cancer is a neoplastic disease which ultimately leads to death of the organism if it is not diagnosed and treated in a timely fashion. Cancer is the second most leading cause of death in the United States, heart disease being first. The 5-year survival rate of people with cancer is still only about 50%. However, it is well known that the survival rate can be greatly enhanced if the cancer is detected at an early stage. Unfortunately there is still a great need for better diagnostic and therapeutic techniques for detecting and treating cancers at an early stage.

SUMMARY OF THE INVENTION

The present invention addresses this need through the discovery that a protein called `secretory protein - 9', hereinafter referred to as `Zsig9' is overexpressed by many tumors. The mature human Zsig9 polypeptide is comprised of a sequence of amino acids approximately 64 amino acids long. Amino acid residue 21 of SEQ ID NO: 2, an arginine, is the initial amino acid of the mature polypeptide. Thus, it is believed that amino residues 1-20 comprise a signal sequence, and the mature Zsig9 polypeptide is represented by the amino acid sequence comprised of residues 21-84. The mature Zsig9 polypeptide is further represented by SEQ ID NO: 3. Alternative forms of Zsig9 are defined by SEQ ID NOs: 4, 5, and 6. SEQ ID NO: 4 defines a processed form of Zsig9 in which the protein contains amino acid residues 23 - 84 of SEQ ID NO: 2.

SEQ ID NO: 5 represents another form of Zsig9 containing amino acid residues 23, a serine, to and including amino acid 47, a proline of SEQ ID NO: 2. SEQ ID NO: 6 defines another processed form of Zsig9 contain amino acid residues 50, a threonine, to and including amino acid 84 of SEQ ID NO: 2. SEQ ID NO: 16 and 17 represent another variant of Zsig9 and SEQ ID NO: 20 represents the mature sequence absent the first 20 amino acid residues, the signal sequence, of SEQ ID NO: 17.

SEQ ID NO: 18 and 19 represent the mouse ortholog of Zsig9; and SEQ ID NO: 21 depicts the mature amino acid sequence absent the first 20 amino acid residues, the signal sequence, of SEQ ID NO: 19.

Thus, the present invention is a method for detecting the presence of tumors in an individual comprised of bringing into contact fluid or cellular material with a labeled antagonist or antibody to Zsig9 under conditions wherein the antagonist or antibody to Zsig9 will bind to Zsig9 present in the fluid or cellular material and detecting said binding.

In another embodiment of the present invention, nucleic acid probes are used to detect cancer cells by testing for the expression of Zsig9 by bringing into contact cellular fluid or other cellular material with nucleic acid probes under conditions wherein the probes bind to ribonucleic acid which encode Zsig9 and detecting said binding.

The present invention further comprises a method for treating a cancer comprising treating an individual with an antagonist to Zsig9 under conditions wherein the antagonist binds to Zsig9 or to a receptor to Zsig9. Preferably the antagonist is an antibody to Zsig9 and preferably the antibody is radiolabeled or fused to a polypeptide toxin.

The present invention is further comprised of a method of treating a cancer comprising administering an anti-sense nucleotide capable of binding to a nucleotide sequence which encodes Zsig9 under conditions wherein the expression of Zsig9 is inhibited.

In a preferred embodiment the tumors which are detected and treated are brain, liver, lung, esophageal, stomach, colon, rectal, thyroid, and lymphoma tumors.

These and other aspects of the invention will become evident upon reference to the following detailed description.

DETAILED DESCRIPTION OF THE INVENTION

The teachings of all of the references cited herein are incorporated in their entirety herein by reference.

The term "receptor" denotes a cell-associated protein that binds to a bioactive molecule (i.e., a ligand) and mediates the effect of the ligand on the cell. Membrane-bound receptors are characterized by a multi-domain structure comprising an extracellular ligand-binding domain and an intracellular effector domain that is typically involved in signal transduction. Binding of ligand to receptor results in a conformational change in the receptor that causes an interaction between the effector domain and other molecule(s) in the cell. This interaction in turn leads to an alteration in the metabolism of the cell. Metabolic events that are linked to receptor-ligand interactions include gene transcription, phosphorylation, dephosphorylation, increases in cyclic AMP production, mobilization of cellular calcium, mobilization of membrane lipids, cell adhesion, hydrolysis of inositol lipids and hydrolysis of phospholipids. Most nuclear receptors also

exhibit a multi-domain structure, including an amino-terminal, transactivating domain, a DNA binding domain and a ligand binding domain. In general, receptors can be membrane bound, cytosolic or nuclear; monomeric (e.g., thyroid stimulating hormone receptor, beta-adrenergic receptor) or multimeric (e.g., PDGF receptor, growth hormone receptor, IL-3 receptor, GM-CSF receptor, G-CSF receptor, erythropoietin receptor and IL-6 receptor).

The term "complement/anti-complement pair" denotes non-identical moieties that form a non-covalently associated, stable pair under appropriate conditions. For instance, biotin and avidin (or streptavidin) are prototypical members of a complement/anti-complement pair. Other exemplary complement/anti-complement pairs include receptor/ligand pairs, antibody/antigen (or hapten or epitope) pairs, sense/antisense polynucleotide pairs, and the like. Where subsequent dissociation of the complement/anti-complement pair is desirable, the complement/anti-complement pair preferably has a binding affinity of $<10^9$ M^{-1}.

Cancer Diagnosis and Therapy

The present invention is a method for diagnosing, staging and treating cancerous tumors. As can be seen in Example 5, Zsig9 is overexpressed in a number of human tumors including brain, liver, lung, esophageal, stomach, colon, rectal, thyroid, and lymphoma tumors. Thus, antagonists, especially antibodies, to Zsig9 can be used both to detect and treat the tumors which overexpress Zsig9. Preferably the antibodies or antagonists are either radiolabeled or fused to a toxic polypeptide.

Nucleotide primers and probes of the *Zsig9* gene can be used to detect the overexpression of Zsig9 using PCR.

Antisense nucleotides to the *Zsig9* DNA and RNA can be administered to a patient to inhibit expression of Zsig9.

Suitable detectable molecules may be directly or indirectly attached to the antagonist or antibody of Zsig9, and include radionuclides, enzymes, substrates, cofactors, inhibitors, fluorescent markers, chemiluminescent markers, magnetic particles and the like. Suitable cytotoxic molecules may be directly or indirectly attached to the polypeptide or antibody, and include bacterial or plant toxins (for instance, diphtheria toxin, *Pseudomonas* exotoxin, ricin, abrin and the like), as well as therapeutic radionuclides, such as iodine-131, rhenium-188 or yttrium-90 (either directly attached to the polypeptide or antibody, or indirectly attached through means of a chelating moiety, for instance). Antagonists or antibodies to Zsig9 may also be conjugated to cytotoxic drugs, such as adriamycin. For indirect attachment of a detectable or cytotoxic molecule, the detectable or cytotoxic molecule may be conjugated with a member of a complementary/ anticomplementary pair, where the other member is bound to the polypeptide or antibody portion. For these purposes, biotin/streptavidin is an exemplary complementary/ anticomplementary pair. The antibody/fragment-toxin fusion proteins may be used for targeted cell or tissue inhibition or ablation (for instance, to treat cancer cells or tissues).

In another embodiment, polypeptide-cytokine fusion proteins or antibody/fragment-cytokine fusion proteins may be used for enhancing *in vitro* cytotoxicity (for instance, that mediated by monoclonal antibodies against tumor targets) and for enhancing *in vivo* killing of target tissues. In general, cytokines are toxic if administered systemically. The described fusion proteins enable targeting of a cytokine to a desired site of action,

thereby providing an elevated local concentration of cytokine. Suitable Zsig9 antagonists or anti-Zsig9 antibodies target an undesirable cell or tissue (i.e., a tumor), and the fused cytokine mediates improved target cell lysis by effector cells. Suitable cytokines for this purpose include interleukin 2 and granulocyte-macrophage colony-stimulating factor (GM-CSF), for instance.

The bioactive polypeptide or antibody conjugates described herein can be delivered intravenously, intraarterially or intraductally, or may be introduced locally at the intended site of action.

Another aspect of the present invention involves antisense polynucleotide compositions that are complementary to a segment of the polynucleotides set forth in SEQ ID NO: 1. Such synthetic antisense oligonucleotides are designed to bind to mRNA encoding Zsig9 polypeptides and to inhibit translation of such mRNA. Such antisense oligonucleotides are used to inhibit expression of Zsig9 polypeptide-encoding genes in cell culture or in a subject. These antisense nucleotides can be used to inhibit the growth of tumors and if labeled to diagnose the presence and stage of tumor growth.

For pharmaceutical use, the antagonists, antibodies and antisense molecules to Zsig9 are formulated for parenteral, particularly intravenous or subcutaneous, delivery according to conventional methods. Intravenous administration will be by bolus injection or infusion over a typical period of one to several hours. In general, pharmaceutical formulations will include an antibody in combination with a pharmaceutically acceptable vehicle, such as saline, buffered saline, 5% dextrose in water or the like. Formulations may further include one or more excipients, preservatives, solubilizers, buffering agents,

albumin to prevent protein loss on vial surfaces, etc. Methods of formulation are well known in the art and are disclosed, for example, in *Remington's Pharmaceutical Sciences*, 19th Edition Gennaro, ed., (Mack Publishing Co., Easton PA, 1995).

Antibodies to the Zsig9 polypeptide can be purified and then administered to a patient. The quantities of reagents necessary for effective therapy will depend upon many different factors, including means of administration, target site, physiological state of the patient, and other medications administered. Thus, treatment dosages should be titrated to optimize safety and efficacy. Typically, dosages used *in vitro* may provide useful guidance in the amounts useful for *in vivo* administration of these reagents. Animal testing of effective doses for treatment of particular disorders will provide further predictive indication of human dosage. Methods for administration include oral, intravenous, peritoneal, intramuscular, or transdermal administration. Pharmaceutically acceptable carriers will include water, saline, buffers to name just a few. Dosage ranges would ordinarily be expected from 1µg to 1000µg per kilogram of body weight per day. However, the doses by be higher or lower as can be determined by a medical doctor with ordinary skill in the art. For a complete discussion of drug formulations and dosage ranges *see Remington's Pharmaceutical Sciences*, 19th Ed., (Mack Publishing Co., Easton, Penn., 1995), and *Goodman and Gilman's: The Pharmacological Bases of Therapeutics*, 9th Ed. (Pergamon Press 1996).

Zsig9 polypeptides can be used to prepare antibodies that specifically bind to Zsig9 epitopes, peptides or polypeptides. The Zsig9 polypeptide or a fragment thereof is inoculated into an animal so as to

elicit an immune response. Antibodies generated from this immune response can be isolated and purified as described herein. Methods for preparing and isolating polyclonal and monoclonal antibodies are well known in the art. See, for example, *Current Protocols in Immunology*, Cooligan, *et al.*, Eds., (National Institutes of Health, John Wiley and Sons, Inc., 1995); Sambrook *et al.*, *Molecular Cloning: A Laboratory Manual, Second Edition*, (Cold Spring Harbor, NY, 1989); and Hurrell, J. G. R., Ed., *Monoclonal Hybridoma Antibodies: Techniques and Applications*, (CRC Press, Inc., Boca Raton, FL, 1982).

As would be evident to one of ordinary skill in the art, polyclonal antibodies can be generated from inoculating a variety of warm-blooded animals such as horses, cows, goats, sheep, dogs, chickens, rabbits, mice, and rats with a Zsig9 polypeptide or a fragment thereof. The immunogenicity of a Zsig9 polypeptide may be increased through the use of an adjuvant, such as alum (aluminum hydroxide) or Freund's complete or incomplete adjuvant. Polypeptides useful for immunization also include fusion polypeptides, such as fusions of Zsig9 or a portion thereof with an immunoglobulin polypeptide or with maltose binding protein. The polypeptide immunogen may be a full-length molecule or a portion thereof. If the polypeptide portion is "hapten-like", such portion may be advantageously joined or linked to a macromolecular carrier (such as keyhole limpet hemocyanin (KLH), bovine serum albumin (BSA) or tetanus toxoid) for immunization.

As used herein, the term "antibodies" includes polyclonal antibodies, affinity-purified polyclonal antibodies, monoclonal antibodies, and antigen-binding fragments, such as $F(ab')_2$ and Fab proteolytic fragments.

Genetically engineered intact antibodies or fragments, such as chimeric antibodies, Fv fragments, single chain antibodies and the like, as well as synthetic antigen-binding peptides and polypeptides, are also included. Non-human antibodies may be humanized by grafting non-human CDRs onto human framework and constant regions, or by incorporating the entire non-human variable domains (optionally "cloaking" them with a human-like surface by replacement of exposed residues, wherein the result is a "veneered" antibody). In some instances, humanized antibodies may retain non-human residues within the human variable region framework domains to enhance proper binding characteristics. Through humanizing antibodies, biological half-life may be increased, and the potential for adverse immune reactions upon administration to humans is reduced.

Alternative techniques for generating or selecting antibodies useful herein include *in vitro* exposure of lymphocytes to Zsig9 protein or peptide, and selection of antibody display libraries in phage or similar vectors (for instance, through use of immobilized or labeled Zsig9 protein or peptide). Genes encoding polypeptides having potential Zsig9 polypeptide binding domains can be obtained by screening random peptide libraries displayed on phage (phage display) or on bacteria, such as *E. coli*. Nucleotide sequences encoding the polypeptides can be obtained in a number of ways, such as through random mutagenesis and random polynucleotide synthesis. These random peptide display libraries can be used to screen for peptides which interact with a known target which can be a protein or polypeptide, such as a ligand or receptor, a biological or synthetic macromolecule, or organic or inorganic substances.

Techniques for creating and screening such random peptide display libraries are known in the art (Ladner *et al.*, US Patent NO. 5,223,409; Ladner *et al.*, US Patent NO. 4,946,778; Ladner *et al.*, US Patent NO. 5,403,484 and Ladner *et al.*, US Patent NO. 5,571,698) and random peptide display libraries and kits for screening such libraries are available commercially, for instance from Clontech (Palo Alto, CA), Invitrogen Inc. (San Diego, CA), New England Biolabs, Inc. (Beverly, MA) and Pharmacia LKB Biotechnology Inc. (Piscataway, NJ). Random peptide display libraries can be screened using the Zsig9 sequences disclosed herein to identify proteins which bind to Zsig9. These "binding proteins" which interact with Zsig9 polypeptides can be directly or indirectly conjugated to drugs, toxins, radionuclides and the like. The binding proteins can also be used for diagnostic assays for determining circulating levels of polypeptides; for detecting or quantitating soluble polypeptides as marker of underlying tumor growth. These binding proteins can also act as Zsig9 "antagonists" to block Zsig9 binding and signal transduction *in vitro* and *in vivo*. These anti-Zsig9 binding proteins would be useful for inhibiting the growth of tumors.

Antibodies are determined to be specifically binding if: 1) they exhibit a threshold level of binding activity, and/or 2) they do not significantly cross-react with related polypeptide molecules. First, antibodies herein specifically bind if they bind to a Zsig9 polypeptide, peptide or epitope with a binding affinity (K_a) of 10^6 M^{-1} or greater, preferably 10^7 M^{-1} or greater, more preferably 10^8 M^{-1} or greater, and most preferably 10^9 M^{-1} or greater. The binding affinity of an antibody can be readily determined by one of ordinary skill in the art, for

example, by Scatchard analysis, Scatchard, G., *Ann. NY Acad. Sci. 51*: 660-672 (1949).

Second, antibodies are determined to specifically bind if they do not significantly cross-react with related polypeptides. Antibodies do not significantly cross-react with related polypeptide molecules, for example, if they detect Zsig9 but not known related polypeptides using a standard Western blot analysis (Ausubel *et al., ibid.*). Moreover, antibodies may be "screened against" known related polypeptides to isolate a population that specifically binds to the inventive polypeptides. For example, antibodies raised to Zsig9 are adsorbed to related polypeptides adhered to insoluble matrix; antibodies specific to Zsig9 will flow through the matrix under the proper buffer conditions. Such screening allows isolation of polyclonal and monoclonal antibodies non-crossreactive to closely related polypeptides, *Antibodies: A Laboratory Manual*, Harlow and Lane (eds.),(Cold Spring Harbor Laboratory Press, 1988); *Current Protocols in Immunology*, Cooligan, *et al.* (eds.), (National Institutes of Health, John Wiley and Sons, Inc., 1995). Screening and isolation of specific antibodies is well known in the art. See, *Fundamental Immunology*, Paul Eds., (Raven Press, 1993); Getzoff *et al., Adv. in Immunol. 43*: 1-98 (1988); *Monoclonal Antibodies: Principles and Practice*, Goding, J.W., Eds., (Academic Press Ltd., 1996); Benjamin *et al., Ann. Rev. Immunol. 2*: 67-101 (1984).

A variety of assays known to those skilled in the art can be utilized to detect antibodies which specifically bind to Zsig9 proteins or peptides. Exemplary assays are described in detail in *Antibodies: A Laboratory Manual*, Harlow and Lane, Eds., (Cold Spring Harbor Laboratory

Press, 1988). Representative examples of such assays include: concurrent immunoelectrophoresis, radioimmunoassay, radioimmuno-precipitation, enzyme-linked immunosorbent assay (ELISA), dot blot or Western blot assay, inhibition or competition assay, and sandwich assay. In addition, antibodies can be screened for binding to wild-type versus mutant Zsig9 protein or polypeptide.

Suitable direct tags or labels include radionuclides, enzymes, substrates, cofactors, inhibitors, fluorescent markers, chemiluminescent markers, magnetic particles and the like; indirect tags or labels may feature use of biotin-avidin or other complement/anti-complement pairs as intermediates. Antibodies herein may also be directly or indirectly conjugated to drugs, toxins, radionuclides and the like, and these conjugates used for *in vivo* diagnostic or therapeutic applications. Moreover, antibodies to Zsig9 or fragments thereof may be used *in vitro* to detect denatured Zsig9 or fragments thereof in assays, for example, Western Blots or other assays known in the art.

As to the selection of peptides or polypeptides bearing an antigenic epitope (*i.e.*, that contain a region of a protein molecule to which an antibody can bind), it is well known in the art that relatively short synthetic peptides that mimic part of a protein sequence are routinely capable of eliciting an antiserum that reacts with the partially mimicked protein. See Sutcliffe, J.G. *et al. Science 219*:660-666 (1983). Peptides capable of eliciting protein-reactive sera are frequently represented in the primary sequence of a protein, can be characterized by a set of simple chemical rules, and are confined neither to immunodominant regions of intact proteins (*i.e.*, immunogenic epitopes) nor to the amino or carboxyl

terminals. Peptides that are extremely hydrophobic and those of six or fewer residues generally are ineffective at inducing antibodies that bind to the mimicked protein; longer soluble peptides, especially those containing proline residues, usually are effective.

Antigenic epitope-bearing peptides and polypeptides of Zsig9 are therefore useful to raise antibodies, including monoclonal antibodies, that bind specifically to a polypeptide of the invention. Antigenic epitope-bearing peptides and polypeptides of the present invention contain a sequence of at least nine, preferably between 15 to about 30 amino acids contained within the amino acid sequence of a polypeptide of the invention. However, peptides or polypeptides comprising a larger portion of an amino acid sequence of the invention, containing from 30 to 50 amino acids, or any length up to and including the entire amino acid sequence of a polypeptide of the invention, also are useful for inducing antibodies that react with the protein. Preferably, the amino acid sequence of the epitope-bearing peptide is selected to provide substantial solubility in aqueous solvents (*i.e.*, the sequence includes relatively hydrophilic residues and hydrophobic residues are preferably avoided); and sequences containing proline residues are particularly preferred.

In another embodiment, antibody-cytokine fusion proteins can be used for enhancing *in vivo* killing of target tissues (for example, blood and bone marrow cancers), if the anti-Zsig9 antibody targets the hyperproliferative blood or bone marrow cell (See, generally, Hornick *et al.*, *Blood* 89:4437-47 (1997). They described fusion proteins enable targeting of a cytokine to a desired site of action, thereby providing an elevated local concentration of cytokine. Suitable anti-Zsig9

antibodies target an undesirable cell or tissue (i.e., a tumor or a leukemia), and the fused cytokine mediated improved target cell lysis by effector cells. Suitable cytokines for this purpose include interleukin 2 and granulocyte-macrophage colony-stimulating factor (GM-CSF), for instance.

The bioactive antibody conjugates described herein can be delivered intravenously, intraarterially or intraductally, or may be introduced locally at the intended site of action.

Those skilled in the art will recognize that the Zsig9 sequences disclosed in SEQ ID NO:1 and SEQ ID NO:2 represent a single allele of the human Zsig9 gene and polypeptide, and that allelic variation and alternative splicing are expected to occur. Allelic variants can be cloned by probing cDNA or genomic libraries from different individuals according to standard procedures. Allelic variants of the DNA sequence shown in SEQ ID NO: 1, including those containing silent mutations and those in which mutations result in amino acid sequence changes, are within the scope of the present invention, as are proteins which are allelic variants of SEQ ID NOs: 3, 4, 5, and 6.

A gene which encode Zsig9 can be synthesized using DNA synthesizers. Currently the method of choice is the phosphoramidite method. Each complementary strand of a double stranded DNA of gene or a gene fragment is made separately. The production of short DNA fragments (60 to 80 bp) can be accomplished by synthesizing the complementary strands and then annealing them. For the production of longer DNA molecules the coupling efficiency of each cycle during chemical DNA synthesis is seldom 100%. To overcome this problem, synthetic genes (double-stranded) are assembled in modular form from single-stranded fragments that are from 20 to 100 nucleotides in length. See Glick,

Bernard R. and Jack J. Pasternak, *Molecular Biotechnology, Principles & Applications of Recombinant DNA*, (ASM Press, Washington, D.C. 1994), Itakura, K. *et al.* Synthesis and use of synthetic oligonucleotides. *Annu. Rev. Biochem. 53* : 323-356 (1984), and Climie, S. *et al.* Chemical synthesis of the thymidylate synthase gene. *Proc. Natl. Acad. Sci. USA 87* :633-637 (1990).

A Zsig9 polypeptide, including full-length proteins, protein fragments (e.g. ligand-binding fragments), and fusion polypeptides can be produced in genetically engineered host cells according to conventional techniques. Suitable host cells are those cell types that can be transformed or transfected with exogenous DNA and grown in culture, and include bacteria, fungal cells, and cultured higher eukaryotic cells. Eukaryotic cells, particularly cultured cells of multicellular organisms, are preferred. Techniques for manipulating cloned DNA molecules and introducing exogenous DNA into a variety of host cells are disclosed by Sambrook *et al.*, *Molecular Cloning: A Laboratory Manual*, 2nd ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, (1989), and Ausubel *et al.*, *ibid*.

In general, a DNA sequence encoding a Zsig9 polypeptide is operably linked to other genetic elements required for its expression, generally including a transcription promoter and terminator, within an expression vector. The vector will also commonly contain one or more selectable markers and one or more origins of replication, although those skilled in the art will recognize that within certain systems selectable markers may be provided on separate vectors, and replication of the exogenous DNA may be provided by integration into the host cell genome.

Selection of promoters, terminators, selectable markers, vectors and other elements is a matter of routine design within the level of ordinary skill in the art. Many such elements are described in the literature and are available through commercial suppliers.

To direct a Zsig9 polypeptide into the secretory pathway of a host cell, a secretory signal sequence (also known as a leader sequence, prepro sequence or pre sequence) is provided in the expression vector. The secretory signal sequence may be that of the protein, or may be derived from another secreted protein (e.g., t-PA) or synthesized *de novo*. The secretory signal sequence is joined to the Zsig9 DNA sequence in the correct reading frame. Secretory signal sequences are commonly positioned 5' to the DNA sequence encoding the polypeptide of interest, although certain signal sequences may be positioned elsewhere in the DNA sequence of interest (see, *e.g.*, Welch *et al.*, U.S. Patent No. 5,037,743; Holland *et al.*, U.S. Patent No. 5,143,830).

Cultured mammalian cells are preferred hosts within the present invention. Methods for introducing exogenous DNA into mammalian host cells include calcium phosphate-mediated transfection, Wigler *et al.*, *Cell* 14:725, (1978); Corsaro and Pearson, *Somatic Cell Genetics* 7:603, (1981): Graham and Van der Eb, *Virology* 52:456, (1973), electroporation, Neumann *et al.*, *EMBO J.* 1:841-845, (1982), DEAE-dextran mediated transfection, Ausubel *et al.*, eds., *Current Protocols in Molecular Biology*, John Wiley and Sons, Inc., NY, (1987), and liposome-mediated transfection, Hawley-Nelson *et al.*, *Focus* 15:73, (1993); Ciccarone *et al.*, *Focus* 15:80, (1993). The production of recombinant polypeptides in cultured mammalian cells is disclosed, for

example, by Levinson *et al.*, U.S. Patent No. 4,713,339; Hagen *et al.*, U.S. Patent No. 4,784,950; Palmiter *et al.*, U.S. Patent No. 4,579,821; and Ringold, U.S. Patent No. 4,656,134. Suitable cultured mammalian cells include the COS-1 (ATCC No. CRL 1650), COS-7 (ATCC No. CRL 1651), BHK (ATCC No. CRL 1632), BHK 570 (ATCC No. CRL 10314), 293, ATCC No. CRL 1573; Graham *et al.*, *J. Gen. Virol.* *36*:59-72, (1977) and Chinese hamster ovary (e.g. CHO-K1; ATCC No. CCL 61) cell lines. Additional suitable cell lines are known in the art and available from public depositories such as the American Type Culture Collection, Rockville, Maryland. In general, strong transcription promoters are preferred, such as promoters from SV-40 or cytomegalovirus. See, *e.g.*, U.S. Patent No. 4,956,288. Other suitable promoters include those from metallothionein genes (U.S. Patent Nos. 4,579,821 and 4,601,978,and the adenovirus major late promoter.

Drug selection is generally used to select for cultured mammalian cells into which foreign DNA has been inserted. Such cells are commonly referred to as "transfectants". Cells that have been cultured in the presence of the selective agent and are able to pass the gene of interest to their progeny are referred to as "stable transfectants." A preferred selectable marker is a gene encoding resistance to the antibiotic neomycin. Selection is carried out in the presence of a neomycin-type drug, such as G-418 or the like. Selection systems may also be used to increase the expression level of the gene of interest, a process referred to as "amplification." Amplification is carried out by culturing transfectants in the presence of a low level of the selective agent and then increasing the amount of selective agent to select for cells that produce high levels of the products of the introduced genes. A preferred amplifiable selectable marker is dihydrofolate reductase, which confers resistance

to methotrexate. Other drug resistance genes (e.g. hygromycin resistance, multi-drug resistance, puromycin acetyltransferase) can also be used.

Other higher eukaryotic cells can also be used as hosts, including insect cells, plant cells and avian cells. Transformation of insect cells and production of foreign polypeptides therein is disclosed by Guarino *et al.*, U.S. Patent No. 5,162,222; Bang *et al.*, U.S. Patent No. 4,775,624; and WIPO publication WO 94/06463. The use of *Agrobacterium rhizogenes* as a vector for expressing genes in plant cells has been reviewed by Sinkar *et al.*, *J. Biosci. (Bangalore) 11*:47-58, (1987).

Fungal cells, including yeast cells, and particularly cells of the genus *Saccharomyces*, can also be used within the present invention, such as for producing protein fragments or polypeptide fusions. Methods for transforming yeast cells with exogenous DNA and producing recombinant polypeptides therefrom are disclosed by, for example, Kawasaki, U.S. Patent No. 4,599,311; Kawasaki *et al.*, U.S. Patent No. 4,931,373; Brake, U.S. Patent No. 4,870,008; Welch *et al.*, U.S. Patent No. 5,037,743; and Murray *et al.*, U.S. Patent No. 4,845,075. Transformed cells are selected by phenotype determined by the selectable marker, commonly drug resistance or the ability to grow in the absence of a particular nutrient (e.g., leucine). A preferred vector system for use in yeast is the *POT1* vector system disclosed by Kawasaki *et al.* (U.S. Patent No. 4,931,373), which allows transformed cells to be selected by growth in glucose-containing media. Suitable promoters and terminators for use in yeast include those from glycolytic enzyme genes (see, e.g., Kawasaki, U.S. Patent No. 4,599,311; Kingsman *et al.*, U.S. Patent No. 4,615,974; and Bitter, U.S. Patent No. 4,977,092) and alcohol

dehydrogenase genes. See also U.S. Patents Nos. 4,990,446; 5,063,154; 5,139,936 and 4,661,454. Transformation systems for other yeasts, including *Hansenula polymorpha, Schizosaccharomyces pombe, Kluyveromyces lactis, Kluyveromyces fragilis, Ustilago maydis, Pichia pastoris, Pichia methanolica, Pichia guillermondii* and *Candida maltosa* are known in the art. See, for example, Gleeson *et al., J. Gen. Microbiol. 132*:3459-3465, (1986) and Cregg, U.S. Patent No. 4,882,279.

Transformed or transfected host cells are cultured according to conventional procedures in a culture medium containing nutrients and other components required for the growth of the chosen host cells. A variety of suitable media, including defined media and complex media, are known in the art and generally include a carbon source, a nitrogen source, essential amino acids, vitamins and minerals. Media may also contain such components as growth factors or serum, as required. The growth medium will generally select for cells containing the exogenously added DNA by, for example, drug selection or deficiency in an essential nutrient which is complemented by the selectable marker carried on the expression vector or co-transfected into the host cell.

PROTEIN ISOLATION:

Expressed recombinant polypeptides (or chimeric polypeptides) can be purified using fractionation and/or conventional purification methods and media. See, for example, *Affinity Chromatography: Principles & Methods*, Pharmacia LKB Biotechnology, Uppsala, Sweden, (1988).

The invention is further illustrated by the following non-limiting examples.

Example 1. Cloning of Zsig9

Zsig9 was identified from expressed sequence tag (EST) SEQ ID NO: 7. The cDNA clone containing the EST was discovered in a placenta from a full-term pregnancy cDNA library which contained the EST. The cDNA was isolated from *E. coli* transfected with the plasmid and then streaked out on an LB 100 µg/ml ampicillin and 100 µg/ml methicillin plate. The cDNA insert was sequenced. The insert was determined to be 649 base pairs long with a 84 amino acid open reading frame and a putative 20 amino acid signal peptide.

Example 2

Construction of Zsig9 Expression Vectors

Two Zsig9 construction vectors were made in a flag amino acid sequence (SEQ ID NO: 8) was inserted onto the N-terminal or C-terminal ends of the Zsig9 polypeptide. For the construction in which the flag amino acid sequence was attached to the N-terminus of Zsig9, a 473 bp Zsig9 PCR DNA fragment was generated with 1 µl of a ¼ dilution of the plasmid prep of Example 1 and 1 microliter (µl) of SEQ ID NO: 9 and 10 each having a concentration of 20 picomoles (pm)/µl of primer. The PCR mixture contained 2.5 µl of 10X PCR buffer,0.5 KLENTAQ (both from CLONTECH), 2.5 µl REDI-LOAD dye (Research Genetics), 2.5 nucleotide triphosphate mix (Perkin-Elmer) and 14 µl of water. The PCR reaction was incubated at 94°C for 5 minutes, and then run for 10 cycles each individual cycle being comprised of 30 seconds at 94°C

and 2 minutes at 75°C. This was followed by 15 cycles each cycle being comprised of 30 seconds at 94°C and 2 minutes at 60°C. The reaction was ended with an incubation for 10 minutes at 74°C.

The resultant PCR mixture was then run on a 0.9% LMP agarose gel with TBE buffer. After the gel was run the band containing the DNA was cut out and the DNA was purified from the gel with a QUIAQUICK® column (Qiagen). 100 µl of the DNA was digested in a solution containing 4 µl of B buffer, 1 µl of *BamH*1 (Boehringer Mannheim) and 1 µl of *Xho*1 (Boehringer Mannheim) for 2 hours at 37°C. The digested reaction mixture was electrophoresed on a 1% TBE gel; the DNA band was excised with a razor blade and the DNA was extracted from the gel with the Qiaquick® Gel Extraction Kit (Qiagen).

The excised DNA was subcloned into plasmid NF/pZP9 which had been cut with *Bam* and *Xho*. NF/pZP9 is a mammalian cell expression vector comprising an expression cassette containing the mouse metallothionein-1 promoter, a sequence encoding the tissue plasminogen activator (TPA) leader, then the flag peptide (SEQ ID NO:8), then multiple restriction sites. These were followed by the human growth hormone terminator, an *E. coli* origin of replication and a mammalian selectable marker expression unit containing the SV40 promoter, enhancer and origin of replication; a dihydrofolate reductase gene (DHFR), and the SV40 terminator. 1 µl containing 10 ng of the NF/pZP9 vector which had been previously digested with *Xho* and *Bam*HI was mixed with 1 µl of 10X ligase buffer, 1 µl of T4 ligase and 2 µl of Zsig9 fragment containing 20 ng. The ligation took place at room temperature for 3 hours and then

electroporated into DH10b cells. After the electroporation the cells were plated onto LB-amp plates.

For the construction of the Zsig9 gene in which the flag polypeptide SEQ ID NO: 8 was inserted onto the C-terminus of the Zsig9 polypeptide, a 649 bp Zsig9 PCR fragment was generated with 1 µl of ¼ dilution of the plasmid preparation containing Zsig9 described in Example 1 and 20 pm each of primers SEQ ID NO: 11 and SEQ ID NO: 12. The PCR reaction was incubated at 94°C for 5 minutes, then run for 10 cycles, each cycle being comprised 30 seconds at 94°C and 2 minutes at 75°C. This was followed by 15 cycles each cycle comprised of 30 seconds at 94°C and 2 minutes at 60°C. The reaction was ended with a final 10 minute extension at 74°C.

The entire reaction mixture was run on a 1% TBE gel and the DNA was cut out with a razor blade and the DNA was extracted using the QIAQUICK™ gel extraction kit. 20 µl out of the recovered 35 µl digested with 10 units of *Bam*H1 (Boehringer Mannheim) and 10 units of *Eco*R1(Gibco BRL) for 2 hours at 37°C. The digested PCR mixture was electrophoresed on a 1% TBE gel. The DNA band was cut out with a razor blade and the DNA was extracted from the gel using the QIAquick® Gel Extraction Kit (Qiagen). The extracted DNA was subcloned into plasmid CF/pZP9 which had been cut with *Eco*R1 and *Bam*H1. Plasmid cfpzp9 is a mammalian expression vector containing an expression cassette having the mouse metallothionein-1 promoter, multiple restriction sites for insertion of coding sequences, a sequence encoding the flag peptide, SEQ ID

NO:10, a stop codon, a human growth hormone terminator, an *E. coli* origin of replication, a mammalian selectable marker expression unit having an SV40 promoter, enhancer and origin of replication, a DHFR gene and the SV40 terminator.

Example 3
Tissue Distribution

Human Multiple Tissue Northern Blots (MTN I, MTN II, and MTN III; Clontech) were probed to determine the tissue distribution of human ZSIG-9 expression. A 40 bp probe (SEQ ID NO: 13) was used to probe the blots. The 5' end of the probe was radioactively labeled using T4 polynucleotide kinase and forward reaction buffer (GIBCO BRL, Gaithersburg, MD) according to the manufacturer's specifications. The probe was purified using a NUCTRAP push column (Stratagene, La Jolla, CA). ExpressHyb™ (Clontech) solution was used for prehybridization and as a hybridizing solution for the Northern blots. Hybridization took place overnight at 42°C using 2 x 10^6 cpm/ml of labeled probe. The blots were then washed at 55°C in 1X SSC, 0.1% SDS. A 1.2 kb transcript was detected. The signal was strongest in heart, placenta, liver and kidney. An intermediate signal was detected in spleen, thymus, prostate, testis, ovary, small intestine, colon, peripheral blood lymphocytes, thyroid, spinal cord. Weak signal was detected in lymph node, trachea, adrenal gland and bone marrow.

EXAMPLE 4

Chromosomal Assignment and Placement of Zsig9.

Zsig9 was mapped to chromosome 12 using the commercially available "GeneBridge 4 Radiation Hybrid Panel" (Research Genetics, Inc., Huntsville, AL). The GeneBridge 4 Radiation Hybrid Panel contains PCRable DNAs from each of 93 radiation hybrid clones, plus two control DNAs (the HFL donor and the A23 recipient). A publicly available WWW server (http://www-genome.wi.mit.edu/cgi-bin/contig/rhmapper.pl) allows mapping relative to the Whitehead Institute/MIT Center for Genome Research's radiation hybrid map of the human genome (the "WICGR" radiation hybrid map) which was constructed with the GeneBridge 4 Radiation Hybrid Panel.

For the mapping of Zsig9 with the "GeneBridge 4 RH Panel", 20 μl reactions were set up in a PCRable 96-well microtiter plate (Stratagene, La Jolla, CA) and used in a "RoboCycler Gradient 96" thermal cycler (Stratagene). Each of the 95 PCR reactions consisted of 2 μl 10X KlenTaq PCR reaction buffer (CLONTECH Laboratories, Inc., Palo Alto, CA), 1.6 μl dNTPs mix (2.5 mM each, PERKIN-ELMER, Foster City, CA), 1 μl sense primer, SEQ ID NO: 14, 1 μl antisense primer, SEQ ID NO: 15, 2 μl "RediLoad" (Research Genetics, Inc., Huntsville, AL), 0.4 μl 50X Advantage KlenTaq Polymerase Mix (Clontech Laboratories, Inc.), 25 ng of DNA from an individual hybrid clone or control and x μl ddH_2O for a total volume of 20 μl. The reactions were overlaid with an equal amount of mineral oil and sealed. The PCR cycler conditions were as follows: an initial 1 cycle 5 minute denaturation at 95°C, 35 cycles of a 1 minute denaturation at 95°C, 1 minute annealing at 62°C and 1.5 minute extension at 72°C, followed by a final 1 cycle extension of 7 minutes at 72°C. The reactions were

separated by electrophoresis on a 3% NuSieve GTG agarose gel (FMC Bioproducts, Rockland, ME).

The results showed that Zsig9 maps 344.72 cR_3000 from the top of the human chromosome 12 linkage group on the WICGR radiation hybrid map. Proximal and distal framework markers were WI-6672 (D12S1410) and RP_L41_1, respectively. The use of the surrounding markers positions Zsig9 in the 12q15 region on the integrated LDB chromosome 12 map (The Genetic Location Database, University of Southhampton, WWW server: http://cedar.genetics. soton.ac.uk/public_html/) .

Example 5
Northern Blot Analysis of Human Tumors

Northern Analysis was carried out on the following Tumor Blots ; Human Tumor Panel Blot I, Human Tumor Panel Blot II, Human Tumor Panel Blot V, Human Stomach Tumor Blot, and Human Colon Tumor Blot (Clontech, Palo Alto, California). A probe was obtained using a PCR product representing the full length coding sequence of zsig9. The probe was radioactively labeled with 32P using Rediprime Labeling System from Amersham (England).The probe was purified using a NUCTRAP push column(Stratagene Cloning Systems, La Jolla, Ca.) . EXPRESSHYB (Clontech, Palo Alto, Ca.) solution was used for prehybridization and hybridization. The hybridization solution consisted of 8 mls EXPRESSHYB, 80 µl Sheared Salmon Sperm DNA (10mg/ml,5 Prime-3 Prime, Boulder, CO) , 48 µl Human Cot-1 DNA (1mg/ml, Gibco BRL) and 20 µl labeled probe (8 x 10-5 CPM/µl). Hybridization took place overnight at 55°C And the blots were then washed in 2X SSC,0.1%SDS at RT, then 2X SSC,0.1% SDS at 60°C, followed by 0.1X SSC, 0.1% SDS wash at 60°C. The blots were exposed overnight and developed.

A transcript of .8-1kb was observed in the following tissues. The strongest signals were in brain tumor, liver tumor, esophageal tumor, stomach tumor, colon tumor, rectal tumor, and thyroid tumor. Weaker signals were in adrenal tumor and normal adrenal, peratoid tumor, and lymphoma tumor. Weakest signals were observed in normal liver, normal esophagus, normal stomach, normal colon, normal rectum, normal thyroid, and normal lymphoma. The Stomach and Colon Tumor Blots showed signals consistent with those observed in the panel blots for stomach and colon tissue.

CLAIMS

We claim:

1. A method for detecting the presence of tumors in an individual comprised of bringing into contact fluid or cellular material with a labeled antibody to Zsig9 under conditions wherein the antagonist or antibody to Zsig9 will bind to Zsig9 present in the fluid or cellular material and detecting said binding.

2. The method of claim 1 wherein the tumors are selected from the group comprising brain, liver, lung, esophageal, stomach, colon, rectal, thyroid, and lymphoma tumors.

3. A method for detecting cancer cells comprising bringing nucleic acid probes into contact with cells, cellular fluid or other bodily fluids under conditions wherein the probes hybridize to ribonucleic acid which encode Zsig9 and detecting said hybridization.

4. The method of claim 3 wherein the tumors are selected from the group comprising brain, liver, lung, esophageal, stomach, colon, rectal, thyroid, and lymphoma tumors.

5. A method for treating a cancer comprising treating an individual with an antagonist to Zsig9.

6. The method of claim 5 wherein the antagonist is radiolabeled or fused to a polypeptide toxin.

7. The method of claim 5 wherein the antagonist is an antibody to either Zsig9 or the receptor to Zsig9.

8. The method of claim 7 wherein the antibody is radiolabeled or fused to a polypeptide toxin.

9. The method of claim 5 wherein the tumors are selected from the group comprising brain, liver, lung, esophageal, stomach, colon, rectal, thyroid, and lymphoma tumors.

10. A method of treating a cancer tumor comprising administering an anti-sense nucleotide capable of binding to a nucleotide sequence which encodes Zsig9 under conditions wherein the expression of Zsig9 is inhibited.

11. The method of claim 10 wherein the tumors are selected from the group comprising brain, liver, lung, esophageal, stomach, colon, rectal, thyroid, and lymphoma tumors.

12. The use of a polynucleotide probe which encodes all or a portion of Zsig9, or an antibody which binds to Zsig9 for detecting the presence of Zsig9.

13. The use of an antagonist to Zsig9 for the treatment of the overexpression of Zsig9.

14. The use of an anti-sense nucleotide capable of binding to a nucleotide sequence which encodes Zsig9 for downregulating Zsig9.

15. The use of antagonist to Zsig9 for the production of a medicament for the treatment of the overexpression of Zsig9.

16. The use of an anti-sense nucleotide capable of binding to a nucleotide sequence which encodes Zsig9 for the production of a mediament for downregulating Zsig9.

SEQUENCE LISTING

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Seattle, Washington 98102
United States of America

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cct tct acc cac cgc aag aac tac gta cgc gtc gtg agc cgg aat gga       696
Pro Ser Thr His Arg Lys Asn Tyr Val Arg Val Val Ser Arg Asn Gly
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gaa tcc agt gaa cta gac tta cag ggc atc cga att gac tca gat atc       744
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gat gag ctt atc gaa ttc ttc tcc aga gag gct gac aac gtt aaa gac      840
Asp Glu Leu Ile Glu Phe Phe Ser Arg Glu Ala Asp Asn Val Lys Asp
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aaa ctt tgc agt aag cgg aca gat cta tgt gac cat gcc ctg cac aga      888
Lys Leu Cys Ser Lys Arg Thr Asp Leu Cys Asp His Ala Leu His Arg
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tct cac gat gag cta tgaatcactg gagcaagcag cctacaccaa acgtgatgga      943
Ser His Asp Glu Leu
        180

acacccccag gaggggaaga tggcagcatt gccttttata ttacgttttt atggaaatga   1003
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            20                  25                  30
Ala Leu Val Asp Glu Leu Glu Trp Glu Ile Ala Arg Val Asp Pro Lys
        35                  40                  45
Lys Thr Ile Gln Met Gly Ser Phe Arg Ile Asn Pro Asp Gly Ser Gln
    50                  55                  60
Ser Val Val Glu Val Pro Tyr Ala Arg Ser Glu Ala His Leu Thr Glu
65                  70                  75                  80
Leu Leu Glu Glu Val Cys Asp Arg Met Lys Glu Tyr Gly Glu Gln Ile
                85                  90                  95
Asp Pro Ser Thr His Arg Lys Asn Tyr Val Arg Val Val Ser Arg Asn
            100                 105                 110
Gly Glu Ser Ser Glu Leu Asp Leu Gln Gly Ile Arg Ile Asp Ser Asp
        115                 120                 125
Ile Ser Gly Thr Leu Lys Phe Ala Cys Glu Ser Ile Val Glu Glu Tyr
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Val Pro Tyr Ala Arg Ser Glu Ala His Leu Thr Glu Leu Leu Glu Glu
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His Arg Lys Asn Tyr Val Arg Val Val Gly Arg Asn Gly Glu Ser Ser
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Glu Leu Asp Leu Gln Gly Ile Arg Ile Asp Ser Asp Ile Ser Gly Thr
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Leu Lys Phe Ala Cys Glu Ser Ile Val Glu Glu Tyr Glu Asp Glu Leu
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Val Pro Tyr Ala Arg Ser Glu Ala His Leu Thr Glu Leu Leu Glu Glu
    50                  55                  60
Val Cys Asp Arg Met Lys Glu Tyr Gly Glu Gln Ile Asp Pro Ser Thr
65                  70                  75                  80
His Arg Lys Asn Tyr Val Arg Val Val Ser Arg Asn Gly Glu Ser Ser
                85                  90                  95
Glu Leu Asp Leu Gln Gly Ile Arg Ile Asp Ser Asp Ile Ser Gly Thr
            100                 105                 110
Leu Lys Phe Ala Cys Glu Ser Ile Val Glu Glu Tyr Glu Asp Glu Leu
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Ile Glu Phe Phe Ser Arg Glu Ala Asp Asn Val Lys Asp Lys Leu Cys
    130                 135                 140
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 1               5                  10                  15
Glu Leu Glu Trp Glu Ile Ala Gln Val Asp Pro Lys Lys Thr Ile Gln
            20                  25                  30
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Met Gly Ser
35

INTERNATIONAL SEARCH REPORT

Inter 'onal Application No
PCT/US 99/11107

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 G01N33/574 C12Q1/68 A61K39/395 A61K48/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 G01N C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	C.-L. HUANG ET AL.: "Two-site monoclonal antibody-based immunoradiometric assay for measuring prostate secretory protein in serum." CLINICAL CHEMISTRY., vol. 38, no. 6, June 1992 (1992-06), pages 817-823, XP002116615 AMERICAN ASSOCIATION FOR CLINICAL CHEMISTRY. WINSTON., US ISSN: 0009-9147 ---	
P,X	WO 99 01554 A (ZYMOGENETICS, INC.) 14 January 1999 (1999-01-14) the whole document -----	1-16

☐ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
27 September 1999	12/10/1999

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Griffith, G

1

INTERNATIONAL SEARCH REPORT

I. national application No.
PCT/US 99/ 11107

Box I Observations where certain claims were found unsearchabl (Continuation of it m 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.: 5-11
because they relate to subject matter not required to be searched by this Authority, namely:
Although claims 5-11 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

2. [ ] Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

Inter ional Application No
PCI/US 99/11107

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9901554 A	14-01-1999	AU 8286698 A	25-01-1999